# Selfed Seed Set and Inbreeding Depression in Obligate Seeding Populations of *Banksia marginata*

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Self-compatible species can often produce seeds when pollinators are scarce or unreliable, but any advantage may be lessened if selfed progeny are less fit than outcrossed progeny due to inbreeding depression. We use hand self-pollinations to determine whether *Banksia marginata* is self-compatible and examine the relative fitness of seeds derived from self- and open-pollination at several early life-cycle stages to gauge the likely impact of inbreeding depression. Substantial numbers of fruits and seeds were produced following selfing, indicating that plants are self-compatible. However, differences between self- and open-pollinated inflorescences indicated that relative self-fertility was less than one. Compared with open-pollinated seeds, selfed seeds were smaller and produced smaller seedlings that were less likely to survive. Percent germination of self- and open-pollinated seeds was similar. Cumulative fitness estimated over several life-cycle stages, including seed production, indicated that selfed progeny were on average only 62% as fit as open-pollinated progeny. These differences in relative fitness indicate that despite self-compatibility, populations have experienced a history of outcrossing. *Banksia marginata* plants at Gibraltar Range National Park are killed by fire, and self-compatibility may be associated with this trait.

## INTRODUCTION

Reproductive assurance is thought to be a powerful selective factor influencing the evolution of self-compatibility in plant populations. Self-compatible species do not require pollen from other plants in order to set seeds and can have an advantage when pollinators are scarce or unreliable (Lloyd 1979, 1992; Barrett 2003). A disadvantage of self-compatibility, however, is that selfed progeny may be less fit than outcrossed progeny due to inbreeding depression (Charlesworth and Charlesworth 1987). One common cause of inbreeding depression is the expression of deleterious recessive alleles made homozygous following selfing. Genetic load and the severity of inbreeding depression are expected to evolve with the mating system. Species with a history of selfing often have low inbreeding depression because deleterious alleles have been purged from the gene pool. By contrast, genetic load is maintained in species that are primarily outcrossing, and inbreeding depression can be severe such that the benefits of self-compatibility are substantially reduced or even negated (Lande et al. 1994; Husband and Schemske 1996; Byers and Waller 1999).

In their review of the breeding and mating systems of the Australian Proteaceae, Goldingay and Carthew (1998) concluded that most *Banksia* species showed only low levels of self-compatibility and were highly outcrossing. Two exceptions were *B. brownii*, which is self-compatible and maintains a mixed mating system with selfing and outcrossing (Sampson et al. 1994, Day et al. 1997), and *B. spinulosa* var. *neoanglica,* which is self-compatible but highly outcrossing (Vaughton 1988; Vaughton and Carthew 1993). Since this review, self-compatibility has been reported in other species of *Banksia* including, *B. ericifolia* var. *macrantha* (Hackett and Goldingay 2001), *B. baxteri*, *B. media* and *B. nutans* (Wooller and Wooller 2001, 2002, 2003). Self-compatibility has also been demonstrated in *B. ilicifolia,* although fruit and seed set following selfing were much lower than following outcrossing (Heliyanto et al. 2005). In two of these species the relative fitness

of selfed progeny was also examined. In *B. baxteri,* more selfed seeds aborted, but seed germination and seedling survival did not differ following self-pollination compared with natural pollination (Wooller and Wooller 2004). In *B. ilicifolia,* fewer selfed seeds germinated than crossed seeds, and survival of selfed seedlings was less when exposed to attack by a fungal pathogen (Heliyanto et al. 2005). Taken together, these results suggest that self-compatibility may be more common in *Banksia* than previously thought, and that in such species the relative fitness of selfed progeny warrants further investigation.

Here we use hand self-pollinations to determine whether *B. marginata* plants occurring at Gibraltar Range National Park (GRNP) are self-compatible. We compare selfed seed set to that occurring naturally in populations and examine variation in the effect of pollination among years and sites. Finally, we assess the relative fitness of seeds derived from self- and open-pollination at several early life-cycle stages to gauge the likely impact of inbreeding depression.

## MATERIALS and METHODS

### Study species and sites

*Banksia marginata* Cav. is widely distributed in south-eastern Australia and exhibits considerable variation in both its morphology and life history throughout its range (George 1998). At GRNP, *B. marginata* is killed by fire and relies on seeds for subsequent regeneration (i.e. plants are obligate seeders, Vaughton and Ramsey 1998; Virgona et al. 2006). Plants occur in sedge-heath in areas of impeded drainage on flats and hillsides (Virgona et al. 2006). Flowering occurs in late autumn and winter and plants produce multiple inflorescences with an average of 784 flowers (SE = 52.4, n = 20). Flowers open acropetally on inflorescences over 3-4 weeks (G. Vaughton unpublished data). Inflorescences are pollinated by nectarivorous honeyeaters, insects, including introduced honeybees, and probably mammals (see methods). Follicles are strongly serotinous and have up to two seeds (Vaughton and Ramsey 1998). Field studies were conducted at two sites within GRNP: Surveyors Creek (SC: 29°32° S, 152°18° E, 1044 m a.s.l) and Waratah Trig (WT: 29°29° S, 152°19° E, 1050 m a.s.l.).

### Self-compatibility

To assess self-compatibility, inflorescences on plants were either bagged and hand self-pollinated or left open to receive natural pollination by pollen

vectors. Either one or two inflorescences at a similar stage of development on each plant were randomly assigned to the two treatments. Selfed inflorescences were covered with nylon mesh bags with apertures of < 1 mm in diameter just prior to flower opening. Every 4-6 days during flowering, bags were removed to self-pollinate flowers and then replaced. We removed pollen from newly opened flowers using pieces of soft cloth attached to small wooden sticks and self-pollinated flowers that had opened a few days previously. When flowering was complete, bags were removed from inflorescences. Open inflorescences were marked with flagging tape but were otherwise left untouched. Cross-pollinations were not performed because we were unable to visit study sites sufficiently often to remove self pollen and thereby avoid possible autonomous self-pollination of flowers. The number of inflorescences developing follicles was scored about 10 months after flowering when follicle development was discernible. Inflorescences with follicles (hereafter cones) were harvested and the numbers of follicles on each were counted. Follicles were opened using a blowtorch and the number of filled seeds per cone was determined.

To assess variation in the natural levels of seed set and the effects of selfing among years and sites, experiments were conducted in three consecutive years at SC (1997, 1998 and 1999) and at SC and WT in 1999. Sample sizes ranged between 12 and 30 plants per year per site but were reduced for final analyses because mammals broke into some bags and cockatoos destroyed some cones before they could be harvested. For the plants with two cones per treatment, the mean value was used in the analyses. Different plants were used in each of the three years at SC. All plants had surplus inflorescences that were not used in the experiment. Pollination treatments were conducted on the same plant to control for plant genotype when assessing seed fitness.

To examine the effect of pollination on the number of inflorescences that produced follicles, we used a logit model with a binomial error term and a logit link function. The response variable was the number of inflorescences with follicles. Explanatory variables were pollination treatment and year or site. Numbers of follicles and seeds per cone were compared between treatments with two-way ANOVAs with pollination treatment as a fixed factor and year or site as random factors. The interaction between the main factors was examined in preliminary analyses and, if not significant ($P > 0.20$), was omitted from the final model to increase the degrees of freedom for testing the main effects. When the interaction was significant,

20

Proc. Linn. Soc. N.S.W., 127, 2006

Table 1. The percentage of *Banksia marginata* inflorescences producing follicles (i.e. cones) and the mean (± SE) numbers of follicles and seeds per cone following either experimental self- or natural open-pollination. The number of plants in each treatment is given in parentheses.

| Trait | Year | Site | Self-pollinated | Open-pollinated |
|---|---|---|---|---|
| Inflorescences with follicles (%) | 1997 | SC | 94 (16) | 92 (25) |
| | 1998 | SC | 94 (35) | 94 (36) |
| | 1999 | SC | 83 (57) | 93 (45) |
| | 1999 | WT | 85 (20) | 89 (44) |
| Number of follicles per cone | 1997 | SC | 22.7 ± 2.4 (15) | 33.3 ± 1.2 (15) |
| | 1998 | SC | 23.0 ± 1.9 (20) | 24.0 ± 1.4 (20) |
| | 1999 | SC | 22.6 ± 2.8 (12) | 22.6 ± 2.8 (12) |
| | 1999 | WT | 19.6 ± 2.4 (12) | 21.0 ± 1.9 (12) |
| Number of seeds per cone | 1997 | SC | 32.1 ± 3.4 (15) | 55.9 ± 1.9 (15) |
| | 1998 | SC | 33.6 ± 2.5 (20) | 46.1 ± 3.7 (20) |
| | 1999 | SC | 26.4 ± 5.0 (12) | 33.3 ± 4.8 (12) |
| | 1999 | WT | 31.0 ± 5.3 (12) | 35.0 ± 4.3 (12) |

differences between the pollination treatments were examined separately for each year or site.

**Progeny fitness**

Seeds produced by self-pollinated inflorescences were self-fertilised, whereas seeds produced by open inflorescences may have been either self- or cross-fertilised. Progeny fitness was examined using a subset of 16 plants at SC in 1998. Seed mass was examined by weighing 20 seeds individually from selfed and open inflorescences on each plant to the nearest 0.1 mg. Individual seeds were placed on the soil surface of tubes (282 cm$^3$) containing a 1:1:1 mixture of sand, loam and peat. Tubes were placed on a bench in a laboratory with natural light at about 20$^\circ$ C and kept moist. Seeds were inspected every day and the number that germinated was scored. About four weeks after sowing when most seedlings had produced expanded cotyledons, tubes were relocated to the glasshouse and arranged randomly on benches. Plants were regularly watered and were fertilised once after 10 weeks with 30 ml of half-strength 'Aquasol'. Plants were inspected weekly and mortality recorded. After 12 weeks seedlings were harvested and plant mass (roots + shoots) was determined after drying at 80$^\circ$ C for 3 days.

The effects of pollination on seed germination and seedling survival were assessed with logit models with a binomial error term and a logit link function.

The response variable was either the number of germinated seeds or the number of surviving seedlings. Pollination treatment and maternal plant were explanatory variables. Differences in seed and seedling mass between the treatments were assessed using two-way ANOVAs, with pollination treatment as a fixed factor and maternal plant as a random factor. To satisfy the assumptions of ANOVA, seedling mass was transformed using natural logarithms.

For each trait, we used individual maternal plants to calculate relative fitness as: $Rf = w_S/w_O$, where $w_S$ and $w_O$, are the mean performances of selfed and open progeny, respectively (Charlesworth and Charlesworth 1987). Cumulative relative fitness was calculated for each maternal plant as the product of relative fitness values for the number of seeds per cone, percent seed germination, percent seedling survival and seedling mass. These traits were chosen because they are related to overall fitness and are probably independent of each other.

## RESULTS

**Self-compatibility**

Over three years at SC at least 83% of inflorescences in both pollination treatments produced follicles (Table 1). The number of inflorescences producing follicles was not dependent on pollination

Proc. Linn. Soc. N.S.W., 127, 2006

21

**Table 2. Effects of self- and open-pollination on progeny fitness in *Banksia marginata*. Sixteen maternal plants were examined at SC in 1998. Sample sizes are given parentheses. Cumulative relative fitness was calculated as the product of the relative fitness of individual traits except seed mass. Relative fitness estimates were calculated as the mean of the 16 maternal plants.**

| Trait | Self-pollinated | Open-pollinated | Relative fitness |
|---|---|---|---|
| Number of seeds per cone | $34.7 \pm 2.9$ (16) | $49.1 \pm 4.2$ (16) | $0.73 \pm 0.05$ |
| Seed mass (mg) | $6.24 \pm 0.10$ (320) | $7.32 \pm 0.07$ (320) | $0.85 \pm 0.03$ |
| Seed germination (%) | 97.2 (320) | 97.8 (320) | $0.99 \pm 0.01$ |
| Seedling survival (%) | 79.1 (311) | 84.0 (313) | $0.95 \pm 0.04$ |
| Seedling mass (mg) | $156.2 \pm 2.9$ (246) | $174.7 \pm 3.3$ (263) | $0.90 \pm 0.03$ |
| Cumulative relative fitness | | | $0.62 \pm 0.05$ |

treatment ($G = 2.96$, df $= 2$, $P = 0.227$), year ($G = 1.44$, df $= 1$, $P = 0.231$), or their interaction ($G = 1.31$, df $= 2$, $P = 0.518$). Differences in the numbers of follicles and seeds per cone between the treatments varied among years as indicated by significant treatment x year interactions and were compared for each year separately (Table 1; treatment x year: follicles, $F_{2,88} = 4.28$, $P = 0.017$; seeds $F_{2,88} = 2.71$, $P = 0.074$). Selfed cones produced significantly fewer follicles than open cones in 1997; differences in other years were not significant (1997, $F_{1,28} = 15.58$, $P < 0.001$; 1998, $F_{1,38} = 0.22$, $P = 0.638$; 1999, $F_{1,22} = 0.10$, $P = 0.758$). In addition, selfed cones produced significantly fewer seeds than open cones in 1997 and 1998, but not 1999 (1997, $F_{1,28} = 37.20$, $P < 0.001$; 1998, $F_{1,38} = 7.80$, $P = 0.008$; 1999; $F_{1,22} = 0.97$, $P = 0.336$).

In 1999 at SC and WT, 85-93% of selfed and open inflorescences produced follicles (Table 1). The number of inflorescences with follicles was not dependent on pollination treatment ($G = 0.004$, df $= 1$, $P = 0.944$), site ($G = 2.34$, df $= 1$, $P = 0.126$), or their interaction ($G = 0.53$, df $= 1$, $P = 0.468$). For the numbers of follicles and seeds per cone, treatment x site interactions were not significant and were removed from the final models (both, $F_{1,44} \leq 0.26$, $P > 0.614$). Numbers of follicles and seeds per cone did not differ between treatments or sites (Table 1, all $F_{1,45} \leq 1.24$, $P > 0.272$).

**Progeny fitness**

Seed and seedling mass were significantly less following self-pollination than open pollination (Tables 2, 3). For seed mass, the significant treatment x plant interaction indicated that selfing negatively affected seed mass to a greater extent in some plants than others. The treatment x plant interaction was marginally significant for seedling mass and was probably related to variation in seed mass. Variation

**Table 3. Results of two-way ANOVAs (F) for seed and seedling mass, and analyses of deviance (G) for seed germination and seedling survival. The effects of self- and open-pollination, maternal plant and their interaction on progeny fitness were examined in *Banksia marginata*. Data are presented in Table 2. †P < 0.08; ** P < 0.01; *** P < 0.001.**

| Trait | Treatment | | Plant | | Interaction | |
|---|---|---|---|---|---|---|
| | df | F or G | df | F or G | df | F or G |
| Seed mass | 1, 15 | 23.31*** | 15, 608 | 29.76*** | 15, 608 | 5.98*** |
| Seedling mass | 1, 15 | 13.00** | 15, 477 | 8.52*** | 15, 477 | 1.58† |
| Seed germination | 1 | 0.26 | 15 | 11.29 | 15 | 16.72 |
| Seedling survival | 1 | 3.27† | 15 | 56.83*** | 15 | 21.77 |

22

Proc. Linn. Soc. N.S.W., 127, 2006

occurring among maternal plants was significant in both analyses (Table 3). Seed germination was independent of pollination treatment, but there was a marginally significant trend for lower survival of selfed progeny compared with open progeny (Tables 2, 3). The treatment x plant interaction was not significant for either trait. Seedling survival, but not seed germination, differed among maternal plants (Table 3).

Relative fitness of selfed versus open progeny for the 16 plants varied from 0.73 for the number of seeds per cone to 0.99 for seed germination (Table 2). Mean cumulative relative fitness estimated from the number of seeds per cone, seed germination, seedling survival and seedling mass was 0.62, indicating that on average selfed progeny were only 62% as fit as open progeny.

## DISCUSSION

Substantial numbers of fruits and seeds were produced following experimental self-pollination, indicating that *Banksia marginata* plants at GRNP are self-compatible. Studies of other banksias have shown that species fall into one of two groups with respect to self-compatibility; those that produce few or no seeds following selfing and those that produce moderate to large numbers of selfed seeds. The results of this study indicate that *B. marginata* should be included in the second group. Other species in this group include *B. spinulosa* var. *neoanglica* (Vaughton 1988), *B. brownii* (Sampson et al. 1994), *B. ericifolia* var. *macrantha* (Hackett and Goldingay 2001) and *B. baxteri* (Wooller and Wooller 2001). Except for *B. spinulosa* var. *neoanglica,* which is able to resprout after fire, *B. marginata* and other *Banksia* species capable of producing high numbers of selfed seeds are killed by fire. The association between self-compatibility and obligate seeding has been noted in other studies of *Banksia* (Sampson et al. 1994; Wooller and Wooller 2001, 2002). Self-compatibility helps to buffer the effects of pollinator scarcity on seed set, and depending on pollinator availability, plants can produce a mixture of selfed and outcrossed seeds, resulting in mixed mating.

Despite the substantial production of selfed seeds in *B. marginata,* differences between selfed and open inflorescences indicate that plants are not completely self-fertile and that some outcrossing occurs under natural conditions. As is common in banksias (Copland and Whelan 1989; Vaughton 1991), fruit and seed set of open inflorescences varied among years and sites, potentially reflecting the availability of pollinators and other factors. At SC in 1997, when follicle and seed production following open pollination were the highest, and hence the least likely to be limited by the availability of cross pollen, open inflorescences produced on average 56 seeds compared with only 33 seeds by self-pollinated inflorescences. If all 56 seeds on open inflorescences were outcrossed, then the maximum relative self-fertility can be estimated by dividing self seed set by open seed set, and would be 0.59. If, however, some of the seeds produced by the open inflorescences were selfed, then maximum crossed seed set is probably greater than 56 seeds. This would provide a lower estimate of self-fertility. Nevertheless, seed set in 1997 must have been close to the maximum because spatial constraints on cones would have limited the production of more follicles. Further, follicles can only produce two seeds, and on average 1.7 seeds per cone were produced on open inflorescences, indicating that our estimate of 0.59 is probably close to the actual relative self-fertility.

The minimum outcrossing rate at SC in 1997 can be estimated if we assume that levels of self-fertilisation on selfed and open inflorescences are similar. Thus, if 33 of the 56 seeds on open inflorescences were self-fertilised, then the remaining 23 seeds would be cross-fertilised, providing an estimated outcrossing rate of 0.41 (i.e. 23/56). The outcrossing rate may have been less in years and sites when selfed and open inflorescences produced similar numbers of seeds. Studies of outcrossing rates in *Banksia* species using genetic markers have generally indicated high outcrossing rates, even for species that exhibit substantial self-fertility. This has been attributed to inbreeding depression and selective abortion of selfed progeny (Vaughton and Carthew 1993; Carthew et al. 1996). An exception is *B. brownii* that appears to maintain lower outcrossing rates than other *Banksia* species (Sampson et al. 1994; Day et al. 1997).

Selfed progeny were less fit than those resulting from open-pollination, indicating that inbreeding depression occurs in *B. marginata.* Compared with open-pollinated seeds, selfed seeds were smaller and produced smaller seedlings that were less likely to survive. Maternal effects could not have been responsible for these differences because we specifically controlled for maternal genotype in our experimental design. Seed mass has been found to be a predictor of seedling size and survival in other plant species (Paz and Martinez-Ramos 2003; Khan 2004). In *B. marginata,* the N and P content of seeds increases linearly with increasing seed mass, rendering seedlings less dependent on external supplies of these nutrients in their natural habitat (Vaughton and Ramsey 1998). In many banksias, seedling establishment occurs after fire and seedlings may utilise N and P reserves in seeds to complement

Proc. Linn. Soc. N.S.W., 127, 2006

23

the high levels of other nutrients that are present in the immediate post-fire environment (Stock et al. 1990).

Cumulative fitness estimated over several life-cycle stages, including the number of seeds per cone, indicated that on average selfed progeny were only 62% as fit as open-pollinated progeny. Assuming all open-pollinated progeny were outcrossed, this equates to moderate inbreeding depression of 0.38. If open inflorescences produced a mixture of selfed and outcrossed progeny, then inbreeding depression would be higher. Fitness differences between the pollination treatments also may have been underestimated in this study because only early life-cycle stages were examined, and plants were grown under benign conditions in the glasshouse (Ramsey and Vaughton 1998). The observed cumulative fitness estimate indicates that despite self-compatibility, these *B. marginata* populations have likely experienced a history of outcrossing. High levels of early-acting inbreeding depression are common in species with substantial outcrossing, and reflect a lack of opportunities for purging deleterious recessive alleles (Husband and Schemske 1996).

Further study of the breeding and mating systems of *B. marginata* is clearly warranted to determine the relative benefits of self-compatibility in providing reproductive assurance and the fitness costs associated with self-pollination. In particular, hand cross-pollinations in combination with self-pollinations would confirm our estimates of relative self-fertility and allow a more accurate estimate of inbreeding depression. Studies using genetic markers would also be valuable in determining realised outcrossing rates in populations and the effects of selfing on population genetic structure. The importance of pollinators for seed set also needs to be determined because banksias have an unusual pollen presentation mechanism, which in some species facilitates autonomous self-pollination and seed set in the absence of pollinators (Vaughton 1988). Finally, *B. marginata* exhibits considerable variation over its geographic range and both obligate seeding and resprouting populations occur (George 1998). Studies of the breeding capabilities of plants over the geographic range, and especially in resprouting populations, may provide insight into the factors favouring the evolution of self-compatibility in this species.

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24

Proc. Linn. Soc. N.S.W., 127, 2006

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Proc. Linn. Soc. N.S.W., 127, 2006

25